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13. ABSTRACT (Maximum 200 Words)

The purpose of this study was to evaluate the combined impact of surgery and immunomodulation with low dose cytoxan and GM-CSF on the development of dendritic cells and the activation of T cells in vivo. MMTV Her2/neu mice, which spontaneously develop mammary tumors were treated with combinations of cytoxan, GM-CSF and surgery. Flow cytometry was used to evaluate blood (B), lymph nodal tissue (LN), and splenocytes (S) for evidence of monocyte differentiation to dendritic cells (DC). Both tumor naïve mice and mice with spontaneous tumor growth were evaluated. With this study we were able to identify a subpopulation of monocytic cells with characteristics consistent with partial differentiation to dendritic cells using the cell surface markers CD11C, MHC II, CD86/CD40, CD80, and Ly6c. While t his study was unable to demonstrate alteration of the cell surface markers of the monocytic cell populations in a manner consistent with dendritic cell differentiation using GM-CSF or cytoxan, this study suggests that the presence of tumor itself may alter the CD40 and MHC II level in the spleen and that the presence of tumor itself may lead to early differentiation of the dendritic cell population in the spleen.

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Final Report for
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The Combined Impact of Surgery and Immunomodulation with Low Dose Cytoxan and GM-CSF in the Early Treatment of Breast Cancer

I. INTRODUCTION: The proposed study uses the MMTV-Her 2/neu transgenic mouse model which spontaneously develops mammary tumors (4)(5). *I hypothesize that: CTX will enhance the immunomodulatory effects of GM-CSF and that DC production will be more dramatic when GM-CSF is administered in close proximity to surgery to remove tumor. Specific aims*: 1. To assess the impact of various combinations of CTX , GM-CSF, and surgery on immune modulation in tumor naïve transgenic Her 2 mice by evaluating the spleen, lymph nodes, and peripheral blood. 2. To assess the impact of various combinations of CTX, GM-CSF, and surgery on immune modulation in Her 2 mice bearing de novo tumors by evaluating the spleen, lymph nodes, and peripheral blood. 3. To determine the impact of combined immune modulation on the development of subsequent recurrent disease as well as metastatic disease in the Her 2 mice. *Immunologic assays* include flow cytometry to evaluate for evidence of DC differentiation and function using markers such as (CD11c+,/CD11b+) (CD11c+/CD8alpha+) (MHC class I or MHC class II) as well as markers for T cells, B cells, and NK cells (Thy1.2, B220, or NK1.1) (2). Spleen and lymph nodes will also be evaluated by immunohistochemistry for the presence of DC cells using a dual detection system of CD11c and CD11b.

II. BODY

Methods/Results:

Surgery (breast biopsy or surgical excision) has been shown previously to stimulate acute inflammation. This initiation of the inflammatory cascade includes the release of chemoattractant proteins in the injured tissue microenvironment, thus recruiting and activating monocytes. In these studies we used the MMTV-Her2/neu transgenic mice to assess the impact of various combinations of cytoxan, GM-CSF, and surgery on immune modulation in the tumor naïve and tumor bearing mice. MMTV-Her2/neu transgenic mice have an intact immune system and spontaneously develop mammary tumors. Because of these characteristics, they were an ideal model to assess the impact of various combinations of cytoxan, GM-CSF, and surgery on immune modulation. Cytoxan previously has been shown to cause a shift from immunosuppression to immunopotentiation when administered prior to surgery as noted by an augmentation of CD11b+ myeloid/macrophages, and enhancement of cytotoxic T lymphocytes. It is for this reason that we tested the use of cytoxan prior to surgical removal of spontaneously growing tumor in MMTV Her2/neu mice. GM-CSF has been shown in vitro to be critical for dendritic cell survival and differentiation. We focused our analysis on the monocyte

derived dendritic cells, as we believe these are the cells most likely to be recruited to the biopsy site and draining lymph nodes. While previous in vitro studies provide a continuous supply of GM-CSF to the monocytes, the precursor cell population, this method of administration is limiting for clinical use in patients. The data reported here examined GM-CSF administered in a pulsatile manner as daily subcutaneous injections. Different schedules were tested as listed below. The presence of antigen is important for dendritic cell function and maturation, thus comparisons were made between mice without tumors and mice with measurable tumors.

Dosing:

Cytoxan (C) was administered at a dose of 10 mg/kg in 0.1 cc via intraperitoneal injection.
GM-CSF (G) was administered at a dose of 1 ug/kg in 0.1 cc via subcutaneous injection.
Injections were performed in tumor bearing mice when the tumors were between 1 and 2 cm in diameter.
Injections in non-tumor bearing mice were matched for age for age with the tumor bearing mice.

Injection schedule

Day 0 represents the day of sacrifice and sample collection for immunologic analysis
T represents the day of tumor biopsy
B represents collection of blood
S represents collection of splenocytes
LN represents collection of draining lymph nodes

Group 1

Treatment			**Injections**	
regimen	**Day -3**	**Day -2**	**Day -1**	**Day 0**
G/C			GM-CSF	T
			CYTOXAN	B,S,LN
G+G+G/C	GM-CSF	GM-CSF	GM-CSF	T
			CYTOXAN	B,S,LN
G+G/C+G	GM-CSF	GM-CSF	GM-CSF	
		CYTOXAN	T	B,S,LN
G+G/C		GM-CSF	GM-CSF	T
			CYTOXAN	B,S,LN
G/C+G		GM-CSF	GM-CSF	
		CYTOXAN	T	B,S,LN

(All mice in group 1 had measurable tumor present at the initiation of injections.)

Group 2a (without tumor)

Treatment regimen	Injections			
	Day -3	**Day -2**	**Day -1**	**Day 0**
G+G/C+G	GM-CSF	GM-CSF CYTOXAN	GM-CSF	B, S, LN
G/C+G		GM-CSF CYTOXAN	GM-CSF	B, S, LN
G/C			GM-CSF CYTOXAN	B, S, LN
G		GM-CSF		B, S, LN
C			CYTOXAN	B, S, LN

Group 2b (with tumor)

Treatment regimen	Injections			
	Day -3	**Day -2**	**Day -1**	**Day 0**
G+G/C+G	GM-CSF	GM-CSF CYTOXAN	GM-CSF T	B, S, LN
G/C+G		GM-CSF CYTOXAN	GM-CSF T	B, S, LN
G/C			GM-CSF CYTOXAN T	B, S, LN
G		GM-CSF	T	B, S, LN
C			CYTOXAN T	B, S, LN

Flow analysis:

In order to determine the role of GM-CSF and cytoxan on *in vivo* monocytes differentiation to dendritic cells (DC), the phenotypic analysis protocol defined by Leon et al (Blood Nov 30, 2003) was used. His studies demonstrated that DC can be derived *in vivo* from monocytes generating CD8- and CD8+ splenic DC (characterized as CD11c+, MHC II- DC precursors.

The population of interest as defined by FSC vs SSC was either a tight lymphocyte region, both the lymphocyte and monocyte/macrophage region, or a more relaxed gate for the dendritic cells as they were more varied in their scatter parameters. The monocyte/dendritic populations were defined by back gating from a double positive population in a dual parameter dot plot that was stained for either the CD11b-PE/Ly6C-FITC for the monocyte gate, CD11c-PE/MHCII-FITC for the dendritic cell gate or a

CD3-PE/CD4-PerCp for lymphocytes. Characterization of monocytes was done by double staining with PE-Conjugated antimouse-CD11b (clone M1/79) and FITC-Conjugated anti mouse-Ly6C (clone AL-21m Pharmingen). Phenotypic analysis of dendritic cells was done using CD11c-PE (clone HL3), CD80-FITC (clone 16-10A1), CD40-FITC (clone HM40-3), CD86-PE (clone GL1), BD Pharmingen) on various tissues such as spleen, axillary lymph nodes, and blood. T cells were characterized by dual staining with PE-conjugated anti-mouse CD3 (clone 17A2), PerCP conjugated CD4 (RM405), APC conjugated anti-mouse CD8a (clone 53-6.7, Pharmingen).

Table 1:
Interpretation of the differentiation of the monocytes populations is based on Leon et al , 2004.

monocytes	CD11C-	MHC II-	CD86-/ CD40-	$F4/80^{LOW}$	$LY\text{-}6C^{HIGH}$
activated monocytes	$CD11C^{low}$	MHC II-	$CD86^{int}$/ $CD40^{int}$	$F4/80^{int}$	$LY\text{-}6C^{HIGH}$
early immature DC's	$CD11C^{high}$	$MHC\ II^{int}$	$CD86^{int}$/ $CD40^{int}$	$F4/80^{int}$	$LY\text{-}6C^{HIGH}$
immature DC's	$CD11C^{high}$	$MHC\ II^{int}$	$CD86^{int}$/ $CD40^{int}$	$F4/80^{int}$	$LY\text{-}6C^{-}$
mature DC's	$CD11C^{high}$	$MHC\ II^{high}$	$CD86^{high}$/ $CD40^{high}$	$F4/80^{int}$	$LY\text{-}6C^{-}$

Figure 1:

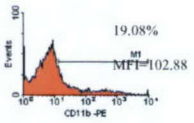
Histograms demonstrate typical staining patterns with CD11b and Ly6C on lymph node specimens obtained from mice treated with either a)cytoxan alone (D-1),b)GM-CSF (D-3), GM-CSF and cytoxan (CTX)(D-1), c) GM-CSF alone, d) GM-CSF (D-2) GMCSF and CTX (D-1) and GM-CSF (D-1).

LYMPH NODE

a) CTX

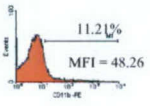
b) GM-CSF and (CTX) +GM-CSF

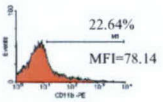
TUMOR



CD11b

NON-TUMOR

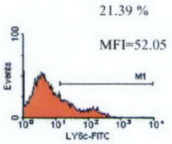




c) GM-CSF

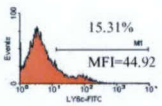
d) GM-CSF+GM-CSF and (CTX) +GM-CSF

TUMOR



Ly6C

NON-TUMOR



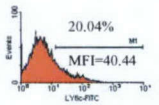
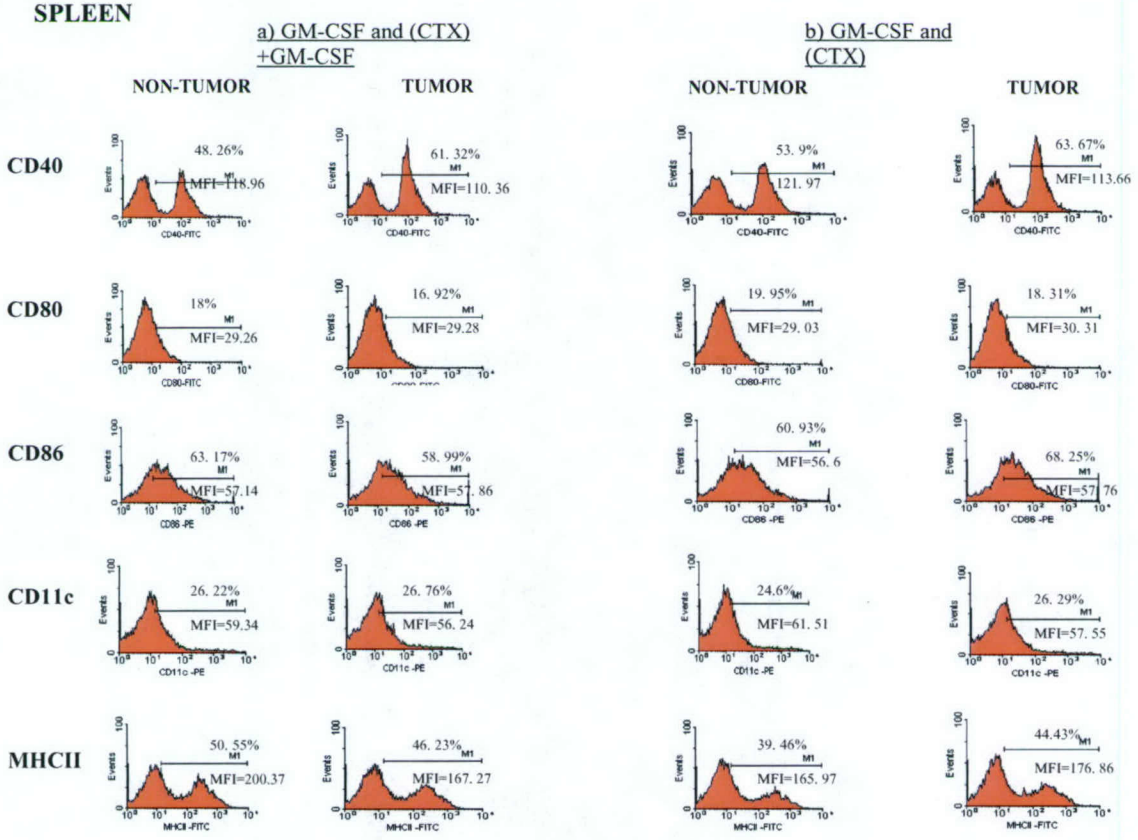


Figure 2:

Histograms demonstrate typical staining patterns from splenocytes obtained from mice treated with either a) GM-CSF and CTX (D-2), GM-CSF (D-1) or b) GM-CSF and CTX (D0-1).



Tables 2a-2f demonstrate the flow cytometry results from blood, lymph nodes, or splenocytes collected from either mice without measurable tumor, or mice with measurable tumor. Surgeries to remove the tumor were performed 24 hours prior to sacrifice of the mice. Treatment regimens used are listed in the first column of each table. The monocytic/dendritic cell populations were defined by back gating from a double positive population in a dual parameter dot plot that was stained for CD11b-PE/Ly6C-FITC or CD11c-PE/MHCII-FITC. Numbers listed on the table are percentage of cells staining positive for the marker listed/ mean fluorescent intensity (MFI). The specific antibodies used to stain the samples are as listed. The antibodies staining used for flow cytometric analysis is based on the characterization of monocyte to dendritic cell maturation/differentiation based on cell surface markers (table 1, page 4).

Table 2a: CD11c

Treatment regimen	Blood		Lymph nodes		Spleen	
	Tumor removed	No tumor	Tumor removed	No tumor	Tumor removed	No tumor
G+G/C+G	9/75	7/31	10/13	12/13	24/56	27/62
G/C+G	26/79	5/46	13/13	13/14	26/56	28/62
G/C	17/37	7/60	13/13	11/13	26/55	27/59
G	33/56	5/50	17/14	10/13	34/62	28/64
C		11/71		12/13		26/60

Table 2b: MHC II

Treatment regimen	Blood		Lymph nodes		Spleen	
	Tumor removed	No tumor	Tumor removed	No tumor	Tumor removed	No tumor
G+G/C+G	9/34	11/258	34/75	44/53	24/56	46/190
G/C+G	16/116	9/201	27/61	38/81	26/56	52/195
G/C	14/133	16/265	31/71	40/61	26/55	42/166
G	29/139	11/246	54/126	30/71	34/62	49/206
C		11/206		33/63		44/182

Table 2c:LY6C

Treatment regimen	Blood		Lymph nodes	
	Tumor removed	No tumor	Tumor removed	No tumor
G+G/C+G	31/28	33/44	14/45	19/40
G/C+G	27/33	37/57	21/52	17/40
G/C	31/30	36/47	22/45	18/42
G	51/39	41/52	21/72	15/45
C		41/38		13/40

Table 2d: CD40

Treatment regimen	Lymph nodes		Spleen	
	Tumor removed	No tumor	Tumor removed	No tumor
G+G/C+G	10/13	12/13	65/117	63/145
G/C+G	13/13	13/14	61/110	54/123
G/C	13/13	11/13	60/112	58/127
G	17/14	10/13	72/109	63/126
C		12/13		62/130

Table 2e: CD80

Treatment regimen	Lymph nodes		Spleen	
	Tumor removed	No tumor	Tumor removed	No tumor
G+G/C+G	5/41	6/36	17/29	19/32
G/C+G	8/52	8/35	17/29	19/29
G/C	7/42	7/36	19/33	18/28
G	11/44	5/28	17/29	18/29
C		6/41		16/28

Table 2f: CD86

Treatment regimen	Lymph nodes		Spleen	
	Tumor removed	No tumor	Tumor removed	No tumor
G+G/C+G	78/44	80/51	61/57	67/61
G/C+G	72/45	83/54	58/58	62/57
G/C	77/46	82/48	65/56	63/57
G	11/44	78/46	61/54	58/56
C		79/45		66/58

The data presented on tables 3a and 3b is derived from the data listed on tables 2 a-f.

Table 3

a) Intensity of staining

Without tumor	*Blood*	*Lymph Nodes*	*Spleen*
CD11c	intermediate	low	intermediate
MHC II	high	low	intermediate
Ly6C	Low	Low	Low
CD40		Low	High
CD80		Low	low
CD86		Low	low

With tumor	*Blood*	*Lymph Nodes*	*Spleen*
CD11c	intermediate	low	intermediate
MHC II	high	low	Low
Ly6C	Low	Low	Low
CD40		Low	High
CD80		Low	low
CD86		Low	low

b) Percentage of positive cells

Without tumor	*Blood*	*Lymph Nodes*	*Spleen*
CD11c	Low	Intermediate	high
MHC II	Low	High	high
Ly6C		High	low
CD40		Low	high
CD80		Low	intermediate
CD86		High	intermediate

With tumor	*Blood*	*Lymph Nodes*	*Spleen*
CD11c	High	Intermediate	high
MHC II	Low	Intermediate	intermediate
Ly6C		Intermediate	intermediate
CD40		Low	high
CD80		Low	intermediate
CD86		High	intermediate

The data listed on tables 4a and 4b represents the lymphocyte population. The mice were treated with either GM-CSF, cytoxan, or a combination as listed in column 1 of the tables. The mice were either tumor naïve or treated with the tumor in place. The tumors were removed 24 hours prior to sacrifice of the mice. The data presented on the table are the percentage of positive cells/ the mean fluorescent intensity. The lymphocytes population was defined by gating for the CD3-PE/CD4-PerCp positive cells.

Table 4a:

Blood

Treatment	CD3		CD4		CD8	
regimen	Tumor removed	No tumor	Tumor removed	No tumor	Tumor removed	No tumor
G+G/C+G	77/221	77/222	64/318	61/313	15/369	16/377
G/C+G	54/258	82/257	51/211	70/328	11/313	14/420
G/C	59/164	75/224	51/254	63/337	11/307	13/319
G	40/154	74/251	37/146	62/320	10/139	14/377
C		77/198		62/295		13/461

Table 4b:

Lymph nodes

Treatment	CD3		CD4		CD8	
regimen	Tumor removed	No tumor	Tumor removed	No tumor	Tumor removed	No tumor
G+G/C+G	85/248	91/412	67/315	77/321	21/231	24/255
G/C+G	76/352	99/239	63/274	76/309	20/300	23/247
G/C	80/174	88/242	64/325	73/322	21/246	2/247
G	63/201	82/196	43/162	67/325	20/289	21/246
C		84/251		65/646		20/241

The data presented on table 5a and b has been extrapolated from the data listed on table 2 and is listed as the mean fluorescent intensity. The cell surface markers used were CD11c, MHC II, CD86, CD40, CD80, and Ly6c. Each sub-table is a specific treatment as listed. The injections schedule is listed on page 6.

Table 5a: Mean Fluorescent Intensity
MICE WITH TUMOR

G + G/C + G

	CD11c	MHC II	CD86	CD40	CD80	LY6C
BLOOD	75	34				
LYMPH NODES	13	75	44	13	41	28
SPLEEN	56	56	57	117	29	45

G/C + G

	CD11c	MHC II	CD86	CD40	CD80	LY6C
BLOOD	79	116				
LYMPH NODES	13	61	45	13	52	33
SPLEEN	56	56	58	110	29	52

G/C

	CD11c	MHC II	CD86	CD40	CD80	LY6C
BLOOD	37	133				
LYMPH NODES	13	71	46	13	42	30
SPLEEN	55	55	56	112	33	45

G

	CD11c	MHC II	CD86	CD40	CD80	LY6C
BLOOD	56	139				
LYMPH NODES	14	126	44	14	44	39
SPLEEN	62	62	54	109	29	72

C

	CD11c	MHC II	CD86	CD40	CD80	LY6C
BLOOD						
LYMPH NODES						
SPLEEN						

Table 5b: Mean Fluorescent Intensity
MICE WITHOUT TUMOR

G + G/C + G

	CD11c	MHC II	CD86	CD40	CD80	LY6C
BLOOD	31	258				
LYMPH NODES	13	53	51	13	36	44
SPLEEN	62	190	61	145	32	40

G/C + G

	CD11c	MHC II	CD86	CD40	CD80	LY6C
BLOOD	46	201				
LYMPH NODES	14	81	54	14	35	57
SPLEEN	62	195	57	123	29	40

G/C

	CD11c	MHC II	CD86	CD40	CD80	LY6C
BLOOD	60	265				
LYMPH NODES	13	61	48	13	36	47
SPLEEN	59	166	57	127	28	42

G

	CD11c	MHC II	CD86	CD40	CD80	LY6C
BLOOD	50	246				
LYMPH NODES	113	71	46	13	28	52
SPLEEN	64	206	56	126	29	45

C

	CD11c	MHC II	CD86	CD40	CD80	LY6C
BLOOD	71	206				
LYMPH NODES	13	63	45	13	41	38
SPLEEN	60	182	58	130	28	40

The data presented on table 6a and b has been extrapolated from the data listed on table 2 and is listed as the percentage of positive cells. The cell surface markers used were CD11c, MHC II, CD86, CD40, CD80, and Ly6c. Each sub-table is a specific treatment as listed. The injections schedule is listed on page 6.

Table 6a: Percentage of positive cells

MICE WITH TUMOR

G + G/C + G

	CD11c	MHC II	CD86	CD40	CD80	LY6C
BLOOD	9	9				
LYMPH NODES	10	34	78	10	5	31
SPLEEN	24	24	61	65	17	14

G/C + G

	CD11c	MHC II	CD86	CD40	CD80	LY6C
BLOOD	26	16				
LYMPH NODES	13	27	72	13	8	27
SPLEEN	26	26	58	61	17	21

G/C

	CD11c	MHC II	CD86	CD40	CD80	LY6C
BLOOD	17	14				
LYMPH NODES	13	31	77	13	7	31
SPLEEN	26	26	65	60	19	22

G

	CD11c	MHC II	CD86	CD40	CD80	LY6C
BLOOD	33	29				
LYMPH NODES	17	54	11	17	11	51
SPLEEN	34	34	61	72	17	21

Table 6b: Percentage of positive cells
MICE WITHOUT TUMOR

G + G/C + G

	CD11c	MHC II	CD86	CD40	CD80	LY6C
BLOOD	7	11				
LYMPH NODES	12	44	80	12	6	33
SPLEEN	27	24	67	63	19	19

G/C + G

	CD11c	MHC II	CD86	CD40	CD80	LY6C
BLOOD	5	9				
LYMPH NODES	133	38	83	13	8	37
SPLEEN	28	52	62	54	19	17

G/C

	CD11c	MHC II	CD86	CD40	CD80	LY6C
BLOOD	7	16				
LYMPH NODES	11	40	82	11	7	36
SPLEEN	27	42	63	58	18	18

G

	CD11c	MHC II	CD86	CD40	CD80	LY6C
BLOOD	5	11				
LYMPH NODES	10	30	78	10	5	41
SPLEEN	28	49	58	63	18	15

C

	CD11c	MHC II	CD86	CD40	CD80	LY6C
BLOOD	11	11				
LYMPH NODES	12	33	79	12	6	41
SPLEEN	26	44	66	62	16	13

Discussion

In conclusion, we were able to identify a subpopulation of monocytic cells with characteristics consistent with partial differentiation to dendritic cells (CD11c, MHC II, CD86/CD40, CD80, Ly6c) in the blood, lymph nodes and spleen of MMTV Her/2neu mice. The presence of tumor did not alter the cell surface marker profile (summary figure A). The presence of tumor did appear to decrease the percentage of MHC II and Ly6C cells in the lymph nodes when compared to the mice without tumor (summary table B). The presence of tumor resulted in a decrease in the percentage of MHC II+ cells and an increase in the Ly6C + cells in the spleen.

Treatment with GM-CSF, in any schedule, resulted in an increase in the size of the lymph nodes (photographs not included).

Characterization of the monocytic populations in the lymph nodes, based on table 1, pg 7, suggest that the monocytic cell population in the lymph nodes does not show evidence of dendritic cell differentiation but more consistent with monocyte activation. Treatment with cytoxan or any of the GM-CSF schedules does not appear to alter the cell surface marker profile as one might expect if dendritic cell differentiation was occurring. The one exception is the lymph nodes collected from mice treated with a solitary dose of GM-CSF. In these mice the MHC II was slightly increased. Cell surface marker profiles in the monocytic cell populations obtained from lymph node harvests are similar in mice with measurable tumors and those without measurable tumors suggesting that if dendritic cell differentiation is occurring in these lymph nodes it is not detectable with the system used in the current study.

Monocytic cell preparations harvested from the spleens of the MMTV Her2/neu mice have a slightly different cell surface marker profile than that seen in the lymph nodes. The CD40 and MHC II levels appear higher in the tumor naïve mice. Using the characterization table on page 7, the profiles demonstrated are consistent with the monocytic populations in the spleen being early immature dendritic cells in the mice bearing tumor and immature dendritic cells in the mice with no measurable tumor. Treatment with cytoxan or any of the GM-CSF schedules does not appear to alter the cell surface profiles, suggesting that such treatment does not differentiate the monocytic cell populations to the extent that it is detectable within the flow cytometric analysis used in this study.

The percentage of CD3+ cells detectable in the blood is not significantly altered by the presence of tumor or treatment with GM-CSF and or cytoxan (table 3a and 3b). There is a trend to a slight decrease in the CD3+ cell population in the lymph nodes with the tumor bearing mice. The percentage of CD8+ and CD4+ cells in the blood and lymph nodes is not altered by the treatment strategies examined or the presence or absence of tumor.

While this study was unable to demonstrate alteration of the cell surface markers of the monocytic cell populations in a manner consistent with dendritic cell differentiation using GM-CSF or cytoxan, this study suggests that the presence of tumor itself may alter the CD40 level on the monocytic cell populations in the spleen (table summary A) and that the presence of tumor itself may lead to early differentiation of the dendritic cell population in the spleen (tables 4 and 5).

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III. KEY RESEARCH ACCOMPLISHMENT:

- Treatment of MMTV Her2/neu mice with GM-CSF results in an increased size of the axillary/inguinal lymph nodes suggestive of biologic activity at the doses/schedules tested.

- The presence of tumors in MMTV Her2/neu mice resulted in an altered cell surface marker expression pattern in the splenocytes as demonstrated by a decrease in the CD40 and MHC II levels. There was no demonstrable difference in the cell surface expression (CD11c, MHC II, CD40, CD86, CD80, Ly6c) in the lymph nodes from either tumor bearing or non-tumor bearing mice. This suggests that the presence of tumor may lead to early differentiation of the monocytic dendritic cell population in the spleen, but not the lymph nodes.

- Treatment with surgery, GM-CSF, +/- cytoxan did not alter the monocytic cell surface marker profiles (CD11c, MHC II, CD40, CD86, CD80, Ly6c) in the lymph nodes, splenocytes, or blood.

- The percentage of lymphoid cells, positive for CD3+, detectable in the blood, is not significantly altered by the presence of tumor or treatment with GM-CSF +/- cytoxan. Suggesting that the differentiation changes in noted monocytic splenocytes from tumor bearing mice did not have an effect on the percentage of CD3+ lymphoid cells in the blood.

IV. REPORTABLE OUTCOMES

Manuscript in development.

V. CONCLUSION

The presence of tumor has a greater impact on the development of splenic, monocytic dendritic cells (as monitored by the cell surface marker profile) than does the treatment with GM-CSF, cytoxan, and surgery.